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(57) Abstract

We have now discovered that spermine is effective in selectively stimulating the death of mamalian prostate cancer cells *in vivo*, and thus is useful in treating prostate cancer. We have further discovered that spermine is effective when administered orally and exhibits low toxicity. Spermine can be administered to a patient upon discovery or removal of the primary tumor in order to prevent or inhibit metastases. Spermine can further be administered to a patient suffering from metastatic disease.

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TREATMENT OF HUMAN PROSTATE CANCER WITH SPERMINE

BACKGROUND OF THE INVENTION

The present invention is directed to methods of inhibiting the spread of prostate cancer, stimulating the death of prostate tumor cells and the diagnosis and treatment of individuals suffering from prostate cancer. Preferably, the method is used to treat individuals suffering from prostate cancer.

Prostatic carcinoma (androgen independent prostate cancer) is the most prevalent form of cancer in males and the second leading cause of cancer death among older males with clinically evident disease usually occurring after the age of 50 (1). Examination of prostates from individuals at autopsy indicates that occurrence of undetected prostatic carcinoma is substantial and increases with age, eventually reaching >50% for men in their eighties (2). Although the incidence of latent prostatic carcinoma is high, the number of patients who develop large, invasive tumors that spread to secondary sites is relatively small, approximately 8% (3). The low apparent progression rate to metastatic disease may be related to the unusually slow increase in tumor mass of primary prostatic carcinomas (4). The time period from neoplastic transformation to palpable tumor or metastatic disease is believed to be considerable, often spanning decades. The importance of this is highlighted in recent work (3,4) which suggests that metastatic potential in prostatic carcinoma correlates directly with primary tumor size and is usually associated with tumors greater than 5 cm^3 in volume. Thus, the nature of the factors that regulate prostatic

carcinoma expansion at the primary site is of crucial importance in understanding the natural history of this disease and being able to treat it.

Prostatic carcinomas show rapid growth after metastasis to bone, most often to the spine. Previous work has indicated that the high concentration of transferrin in bone marrow may facilitate the growth of prostatic carcinoma metastases at that site (5). This suggests that the slow growth of prostatic carcinoma cells in the primary site may be the result of a number of mechanisms-(a) an intrinsic property of this tumor which is overcome by positive stimulation at the bone, (b) a negative effect of the prostatic micro-environment on carcinoma cell growth in the prostate, (c) combinations thereof.

The present clinical treatment for metastatic disease is typically hormonal therapy, which is not curative. Thus, the metastatic disease is typically fatal.

Hormonal therapy consisting of different approaches to blocking the action of androgen on the prostate tumor is effective in controlling only the growth of tumor cells that depend on androgen for growth (hormone-dependent tumor). Unfortunately, hormone-dependent tumor inevitably progresses to more advanced hormone-independent tumor, which cannot be controlled by current treatment. Difficulties in treating prostate cancer arise from a variety of reasons. Although such androgen ablation is a standard therapy for metastatic prostate cancer it is rarely entirely successful because in most individuals the cancer is heterogeneous comprising both androgen dependent and androgen independent cancer cells.

Thus, the therapy does not eliminate the androgen independent cells.

Chemotherapy, which has been used to treat a number of other cancers, has not proven successful. This is because the vast majority of these androgen independent cells are not actively proliferating and standard chemotherapeutic agents work by selectively killing actively proliferating cells.

Radiation therapy, which also is selective for rapidly proliferating cells, has also not proven effective. Surgery has also not proven an effective means for treating advanced disease states.

Accordingly, it would be desirable to have new methods for stimulating the death of these slow proliferating cancer cells. It would be particularly desirable to have a new means of treating individuals suffering from prostate cancer, particularly metastatic disease.

Smith el al. reported in an abstract presented at the American Association for Cancer Research annual meeting (April, 1994) the isolation of a naturally present low molecular weight substance, spermine, from aqueous extracts of human prostatic tissue that inhibits the growth of prostatic adenocarcinoma cell lines *in vitro*.

Other naturally present cell growth inhibiting factors have been isolated but have not proven effective in the inhibition of cancer cell growth *in vivo*.

Spermine, one of three naturally occurring polyamines, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, is essential for cell growth and

differentiation and is particularly abundant in the prostate. Spermine is commonly used as a biochemical reagent. See, for example, The Merck Index, 11th edition (1989).

SUMMARY OF THE INVENTION

We have now discovered that spermine is effective in selectively stimulating the death of mammalian prostate cancer cells *in vivo*, and thus is useful in treating prostate cancer. We have further discovered that spermine is effective when administered orally and exhibits low toxicity. Spermine can be administered to a patient upon discovery or removal of the primary tumor in order to prevent or inhibit metastases. Spermine can further be administered to a patient suffering from metastatic disease. Spermine can also be administered prophylactically in patients who have a family history of prostate cancer, have a high prostate-specific antigen (PSA) level, e.g., 4 or over, or who have had an increase in PSA levels over any six month period.

The present invention further provides a method for inducing cell apoptosis *in vivo* which comprises contacting the cell with an effective amount of spermine. The cell is preferably a human prostate cell. More preferably, the cell is a human prostate cancer cell.

The present invention also provides a method of treating benign prostate hyperplasia in a human comprising administering to said human an effective treatment amount of spermine.

We have also discovered that locally high concentrations of spermine in the prostate acts to contain prostatic carcinoma cell growth. Thus, systemic treatments that lower polyamine levels may

remove an endogenous brake on the growth of primary prostatic tumors. The present invention provides a method of screening a patient for the presence of a primary prostate tumor prior to the administration of an agent that blocks or inhibits polyamine synthesis, e.g., polyamine analogs. If it is determined that the patient has a primary prostate tumor then the prolonged administration of the agent is contraindicated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, 1C and 1D show the isolation of a prostatic carcinoma inhibitor from aqueous extracts of human prostate. (1A) The prostatic aqueous extracts were filtered through an Amicon PM-10 membrane filter (10,000 molecular weight cutoff, MWCO) utilizing an Amicon stirred pressure cell and the filtrate concentrated using an Amicon YC-05 filter (Approx. 500 MWCO). The YC05 retentate was assayed for growth inhibition using 5 x 10^3 PC-3 cells that were plated on 96 well tissue culture plates in RPMI 1640 medium containing 0.5% fetal bovine serum. One day after plating, the cells were challenged with inhibitor in the presence of 5% FBS. After 3 days incubation, cell numbers were determined using a colorimetric assay that measures reduction of the tetrazolium dye MTT (29). (1B) Cation exchange HPLC of YC-05 retentate on carboxymethyl cellulose (CM300); (1C) Heparin sepharose chromatography of the pooled active fractions from CM300; (1D) Size exclusion HPLC of active fractions from heparin sepharose chromatography. Growth inhibitory activity of column eluates were determined by MTT assay using PC-3 cells grown in medium containing 5% fetal bovine serum (FBS).

Figures 2A and 2B show the identification of purified prostatic carcinoma inhibitor as the common polyamine spermine. (2A) Low

resolution mass spectroscopy of prostatic carcinoma inhibitor utilizing the fast atom bombardment method of ionization and (2B) C18 reversed-phase chromatography of the dansylated derivatives of prostatic carcinoma inhibitor and the common polyamines spermine, spermidine and putrescine.

Figures 3A and 3B show the inhibitory activity of spermine on prostatic carcinoma cell growth. (3A) The growth inhibitory activity of purified prostatic inhibitor (closed circles) on PC-3 human prostatic carcinoma cells was compared with that of commercially available purified spermine (open circles). Spermine concentrations in the two preparations were measured after dansylation utilizing reversed phase HPLC to quantify dansyl-spermine levels. (3B) Time course of PC-3 cell growth in the presence or absence of spermine was determined Cells were plated at 5,000 cells per well of a 24 well culture dish in medium containing 5% FBS. The next day, spermine was added to the cultures at a concentration of 290 mM and cell number was determined over the course of 60 hours.

Figure 4 shows the effect of aminoguanidine on inhibition of prostatic carcinoma proliferation by spermine. The effect of 1 mM aminoguanidine (AMG) on the 5% FBS-stimulated growth of PC-3 cells (left panel) and on the inhibitory activity of spermine in the presence of 5% FBS (right panel). Cell counts were determined 3 days after spermine/AMG addition.

Figure 5 shows *in vitro* effects of spermine and putrescine on AT3.1 rat prostatic carcinoma cell growth. Spermine or putrescine was added to cultures of rat AT3.1 prostatatic carcinoma cells in RPMI medium containing 5% FBS and cell counts were performed three days after polyamine addition.

Figures 6A and 6B show the effect of spermine and putrescine on *in vivo* growth of AT3.1 prostatic carcinoma cells in syngeneic rats. (6A) Copenhagen rats were injected subcutaneously with 5 X 10^3 AT3.1 rat prostatic carcinoma cells. After seven days, when the tumors had become palpable, the animals were treated with daily inoculations of spermine (Sm), putrescine (Pu) or control buffer (PBS). Tumor volumes were estimated in living animals, after measurement of tumor length in two directions. Upon completion of the treatment period the tumors were excised and weighed. The final measured density was used to extrapolate tumor weights for earlier time points using the daily volume measurements. (6B) The same experiment was performed with daily intratumoral inoculations of 100 μl of 0.12 M spermine, 1mM aminoguanidine (AMG) or a mixture of spermine plus AMG in phosphate buffered saline. Data is expressed as mean tumor weight +/- s.e.m. (n = 7-9).

Figure 7 shows inhibition of AT3.1 prostate cancer growth *in vivo*.

Figures 8A and 8B show inhibition of AT3.1 prostate cancer growth *in vivo*. In figure 8A spermine was administered as a single bolus (140 mg/40 ml H_2O) once per day. In figure 8B spermine (140 mg) was administered at will through the drinking water.

DETAILED DESCRIPTION OF THE INVENTION

We have now discovered that spermine is effective in selectively stimulating the death of mammalian prostate cancer cells *in vivo*, and thus is useful in treating prostate cancer. Spermine can be administered to a patient upon discovery or removal of the

primary tumor in order to prevent or inhibit metastases. Spermine can further be administered to a patient suffering from metastatic disease in order to inhibit the growth of the secondary tumors.

Spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ can be isolated from human prostate tissue using, for example, the methods taught herein or obtained commercially, for example, spermine tetrachloride is available from Sigma Chemical Corp. The spermine can be brought to a neutral pH before administration or administered without buffer. As used herein "spermine" includes pharmaceutically acceptable salts thereof.

Based on the present disclosure one can also use derivatives of spermine which exhibit at least 70% of spermine's anti-neoplastic activity. This can be determined by measuring the effect of the derivative on the *in vivo* growth of AT3.1 prostatic carcinoma cells in syngeneic rats as taught herein. Preferably, the derivative should not inhibit the release or action of spermine, or counter polyamine synthesis. Spermine derivatives can be formed using standard techniques well known in the chemical arts.

We demonstrate using a variety of human prostate cancer cells *in vivo* their sensitivity to spermine. We demonstrate that spermine administration, both intratumorally and orally, inhibited human prostate tumor growth. We believe these cells were induced to undergo a process of programmed cell death (apoptosis). Thus, the present invention can be used to provide a method for inducing cell apoptosis *in vivo* which comprises contacting the cell with an effective amount of spermine. The cell is preferably a human prostate cell. More preferably, the cell is a human prostate cancer cell. Spermine also inhibited prostate cell adhesion.

Spermine can be utilized both prophylactically and therapeutically. The amount of spermine for therapeutic administration can vary over a wide range and is dependent upon such factors as age of the patient, weight, nature and severity of the disease being treated. In general, for the treatment of prostate cancer, a suitable therapeutically effective dose of spermine will be preferably in the range of 1 μg to 10g per kilogram body weight of recipient per day, more preferably in the range of 10 mg to 5g per kilogram body weight per day, most preferably in the range of 100 to 1000 mg per kilogram body weight per day. The desired dose is suitably administered once or several more sub-doses administered at appropriate intervals throughout the day, or other appropriate schedule.

Spermine at certain serum concentrations serves to place a check on the growth of a tumor. One can create such a local environment at prostate cancer sites and insure that the spermine level remains above the threshold level to inhibit the spread of tumor growth and its size. We believe that even in the prostate as the tumor grows or the patient ages the spermine levels falls. Thus, monitoring the size of the tumor is extremely important. For example, it is desirable to keep the tumor below 4 cm^3.

Accordingly, one would use an effective amount of spermine in a method to stimulate the death of prostate tumor cells. One would select a subject having prostatic tumor cells and administer an effective amount of spermine to treat the prostatic tumor cell. Preferably, the subject is a human. The tumor cell may be part of a primary tumor or a metastases.

Spermine can be administered as a prophylactic upon discovery or removal of a primary tumor in order to prevent or inhibit metastases.

Administration of the compounds of the invention may be by any suitable route including oral, rectal, nasal, topical (including buccal and sublingual) and parenteral (including subcutaneous, intramuscular, intravenous and intradermal) with oral or parenteral being preferred. It will be appreciated that the preferred route may vary with, for example, the condition and age of the recipient.

We have discovered that spermine can be administered orally, without toxicity.

The administrative ingredients may be used in therapy in conjunction with other medicaments, including, for example, chemotherapeutic agents, antiangiogenesis agents, immuno-therapeutics and cancer vaccines.

While spermine may be administered alone, it also may be present as part of a pharmaceutical composition. The compositions of the invention comprise spermine together with one or more acceptable carriers thereof and optionally other therapeutic ingredients, including those therapeutic agents discussed *supra*. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The compositions include those suitable for oral, rectal, nasal, topical (including buccal and sublingual) or parenteral (including subcutaneous, intramuscular, intravenous and intradermal)

administration. The compositions may conveniently be presented in unit dosage form, e.g., tablets and sustained release capsules, and in liposomes and may be prepared by any methods well known in the art of pharmacy.

Such methods include the step of bringing into association the to be administered ingredients with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, liposomes or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion or packed in liposomes and as a bolus, etc.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein.

Compositions suitable for oral administration include lozenges comprising the ingredients in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the ingredient to be administered in a suitable liquid carrier.

When administered orally, the preferred dosage form is that of a tablet or capsule. The amount of active ingredient contained in each dosage unit will vary depending upon the particular dosage form utilized. Generally, a given dosage unit will contain from 50 μg to 2g of spermine in addition to the various pharmaceutical exipients contained therein. Tablets containing from 50 mg to 1000 mg are the preferred dosage unit.

Compositions suitable for topical administration to the skin may be presented as ointments, creams, gels and pastes comprising spermine and a pharmaceutically acceptable carrier. A suitable topical delivery system is a transdermal patch containing the ingredient to be administered.

Compositions suitable for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Compositions suitable for nasal administration wherein the carrier is a solid include a coarse powder having a particle size, for example, in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid, for

administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

To ensure solubility, the compounds are preferably dissolved in a non-ionic solubilizer such as an ethylene oxide ester-ether and fatty acid glycerides commercially available as Cremphor EL (BASF).

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

In another embodiment, we have discovered a simple screen to be used on males at risk for prostate cancer before administering polyamine analogs. Locally high concentrations of spermine in the prostate acts to contain prostatic carcinoma cell growth. Thus,

systemic treatments that lower polyamine levels may create a new problem by removing an endogenous brake on the growth of primary prostatic tumors. The present invention provides a method for determining whether administration of an agent that blocks or inhibits polyamine synthesis, e.g., polyamine analogs, is an appropriate treatment comprising screening a patient at risk for prostate cancer for the presence of a primary prostate tumor, and if present its size. Test for prostate cancer include the prostate-specific antigen (PSA) test. If it is determined that the patient has a primary prostate tumor then the prolonged administration of the agent is contraindicated.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Methods

Preparation of crude prostatic inhibitor

Disease-free human prostate tissue, frozen at -80°C within 6 hr post mortem, was obtained from the National Disease Resource Interchange, (Phil. PA). The frozen material was processed into fine shavings with a scalpel, thawed and extracted 4 times with equal volumes of deionized water. The supernatants were combined, cleared of debris by centrifugation at 32,000 x g for 20 min., filter sterilized (0.20 mm pore size) and subjected to ultrafiltration through an Amicon PM-10 filter (10,000 MWCO). The filtrate was concentrated 20X on a YC-05 filter (< 500 MWCO) and stored at -80°C until tested.

Cation exchange was performed on a Hitachi L4000 HPLC apparatus utilizing a 10 x 250 mm Synchropak CM300 carboxymethyl cation exchange column (Rainin, Woburn, MA) eluted with a linear gradient of 10 mM to 1.0 M ammonium acetate at a flow rate of 1 ml/min. Five ml fractions were collected, dried under vacuum, resuspended in de-ionized water and taken to dryness again. This process was repeated 4 times. Fractions were filter-sterilized and assayed at 10% (v/v) concentration for inhibition of 5% fetal bovine serum-stimulated growth of PC-3 cells. The major inhibitory activity was eluted as a broad peak at approximately 1.0 M ammonium acetate. Active fractions were pooled and concentrated under vacuum. Pooled active fractions from cation exchange chromatography were further fractionated on 0.8 x 4.0 cm heparin- sepharose column and eluted with a linear gradient (50 ml) of 10 mM to 3 M ammonium acetate at a flow rate of 4 ml/hr. Five ml fractions were dried and assayed for growth inhibition as for cation exchange chromatography. Inhibitory activity eluted at approximately 0.75 M ammonium acetate. Pooled active fractions from heparin sepharose chromatography were fractionated by size exclusion HPLC on a 7.8 x 300 mm Progel TSK G2500PWXL column (Supelco, Bellefonte, PA). The column was eluted at a flow rate of 1 ml/min with a dilute solution of hydrochloric acid (pH 4.4) and the optical density monitored at 210 nm. One ml fractions were taken to dryness under vacuum, resuspended in deionized water, filter-sterilized and assayed at a final dilution of 10% (v/v). A single major peak of inhibitory activity eluted from this column at 8.6 minutes. Purified inhibitor and commercially obtained polyamines were derivitized with dansyl chloride and analyzed on pre-activated silica gel 60 plates (E.M. Merck, Germany) by ascending thin layer chromatography in chloroform:triethylamine (5:1) according to the method of Heby and

Anderson (8). Fluor scent species were visualized by exposure to ultraviolet light following evaporation of the mobile phase. The dansylated derivatives were quantified by reversed phase HPLC using a 4.6 x 250 mm Ultrapore RPMC C_{18} column (Beckman Instruments, Carlsbad, CA). The column was calibrated with dansylated derivatives of spermine, spermidine and putrescine and eluted according to the gradient I method of Kabra et al. (29).

Cell proliferation assay

Cell proliferation was measured using cultured human or rat prostatic carcinoma cells grown in response to 5% fetal bovine serum in RPMI culture medium. Cell number was determined using a tetrazolium-based colorimetric assay (28) or by direct counting of trypsinized cells using a Coulter particle counter.

In vivo tumor growth assays

In vivo experiments were conducted using 200-300 gm Copenhagen rats (Harlan-Sprague Dawley, Minneapolis, MN). The animals were injected subcutaneously on the lower back, using a 28 guage needle, with 5 X 10^3 AT3.1 rat prostatic carcinoma cells in 0.1 ml RPMI medium containing 250 nM dexamethasone to promote cell viability. After seven days, when tumors had become palpable, the animals were treated with daily inoculations of neutralized polyamine in 0.1 ml phosphate buffered saline (PBS) or with PBS alone for an additional 7-8 days. Tumor volumes were estimated in living animals, after measurement of tumor length in two directions, using the formula $V = LW^2/2$. On the final day of the experiment, the tumors were excised and weighed. Data is expressed as tumor weight extrapolated from the tumor density on the final day. In some cases, aminoguanidine (1 mM in PBS) was coinjected in solution with spermine.

R sults.

Isolation of an endogenous prostatic inhibitor.

Prostatic extracts were prepared from frozen tissue provided by the National Disease Research Interchange (NDRI, Philadelphia. PA). Samples from individuals with prostatic carcinoma or prostatic hyperplasia were not included in these studies. The extracts were prepared by extracting (4X) in equal volumes of deionized water with clarification by centrifugation (32,000 x g). When tested for their ability to affect the growth of PC3 human adenocarcinoma cells (Fig. 1A), concentrated prostatic extracts showed substantial inhibitory activity with nearly 90% growth inhibition when added to culture medium at a ratio of 1% (vol/vol). Ultrafiltration studies revealed that the inhibitory activity could permeate an Amicon PM-10 filter (molecular weight cutoff of 10,000) but was retained on a YC-05 filter (nominal cutoff of 500). Consequently, an initial purification step was developed in which the crude prostatic material was passed through a PM-10 filter and subsequently collected and concentrated 20-fold using a YC-05 filter. The retentate was then applied to a CM300 HPLC cation exchange column. As shown in Fig. 1A, the inhibitory activity bound to the column and was eluted with 0.85-1.0 M ammonium acetate. The active material was subsequently bound to a heparin-sepharose affinity column with elution of activity occurring at 0.5-1.0 M ammonium acetate (Fig. 1A). Final purification of the material was accomplished by gel exclusion HPLC using a G2500PWXL column (Fig 1A). The identity of the inhibitor was determined by mass spectroscopy (Fig 2A), revealing a molecular ion (+1) of 203.224 with probable composition of $C_{10}H_{27}N_4$. This corresponds to the composition of spermine, a polyamine known to be abundant in prostatic tissue (6,7).

Confirmation of the inhibitor as spermine.

The identity of the purified inhibitor as spermine was confirmed by dansylation of the purified material followed by passage over C_{18} reversed phase HPLC (8). Fig. 2B reveals that the chromatographic profile of the purified inhibitor mimics that of spermine and not that of other related polyamines. When equivalent concentrations of purified inhibitor and commercial spermine were compared for their ability to inhibit the growth of prostatic carcinoma cells (Fig. 3A), the results were nearly identical with significant inhibitory activity seen at 5-20 μM with either preparation. A time course of spermine action on growth of PC-3 cells in vitro revealed that, even at concentrations (290 μM) that are 10-fold greater than the minimal levels demonstrated to inhibit cell growth, spermine prevented population growth without causing a substantial decrease in the initial cell number over the course of 60 hr (Fig. 3B). This suggests that, for the population *in vitro*, spermine acts as a cytostatic agent.

Spermine has been shown to inhibit the growth of certain cell types, and multiple mechanisms have been proposed to account for this activity (9-12). Polyamine uptake has been shown to reduce cell proliferation by inhibiting endogenous polyamine synthesis (13). Metabolic byproducts of spermine catabolism, including acrolein and hydrogen peroxide, may also cause growth inhibition or cell death (9-12). Formation of these intermediates can be catalyzed by the action of amine oxidases present in mammalian plasma. When we included aminoguanidine, an inhibitor of serum amine oxidases (14), in the spermine-containing medium we observed a loss of spermine inhibitory activity (Fig. 4) at concentrations up to 1.5 mM but at 5.0 mM, inhibition was no longer be blocked by AMG. Thus spermine can inhibit cell growth by either a diamine oxidase-dependent or

independent pathway depending on the concentration in the micro-environment.

Inhibition of subcutaneous tumor growth *in vivo*

To determine whether spermine could inhibit prostatic carcinoma cell growth *in vivo*, we utilized the highly aggressive AT3.1 Dunning rat prostatic carcinoma cell line (15). When implanted subcutaneously in syngeneic Copenhagen rats, these tumors grow rapidly, producing >5 cm^3 tumors within 12 days. Spermine inhibited the *in vitro* growth of AT3.1 carcinoma cells whereas putrescine, a related polyamine, was not inhibitory (Fig. 5). We then tested the effects of spermine and putrescine on AT3.1 tumor growth *in vivo*. Starting on day 8, subcutaneous tumors were injected daily with putrescine, spermine or control buffer. Tumor size was measured daily and the tumor volume estimated from the recorded length and width of each tumor. Tumor weights were determined at the end of the experiment and extrapolated for earlier days on the basis of the measured tumor volume. As shown in Fig. 6A, the intratumoral inoculation of spermine at concentrations of 12 micromoles/tumor/day had a profound inhibitory effect on subcutaneous tumor growth whereas the related polyamine putrescine had no inhibitory activity at the same concentrations. No overt signs of toxicity were noted in animals receiving the intratumoral spermine inoculations.

To determine whether spermine-mediated inhibition of prostatic carcinoma cell growth *in vivo* was amine oxidase-dependent, we coinjected spermine intratumorally along with 1 mM AMG (14). AMG had no significant effect on the inhibitory activity of spermine in this experiment suggesting that, at the spermine concentration employed, spermine inhibits carcinoma cell growth *in*

vivo by an amine oxidase independent mechanism (Fig. 6B). AMG alone, without added spermine was not inhibitory and, unexpectedly, caused an increase in tumor volume over the course of the experiment. The results demonstrate that spermine is a potent inhibitor of carcinoma expansion *in vivo* and that this inhibition is not dependent on spermine metabolism by amine oxidases.

Cell Culture

AT3.1 Dunning Rat Prostatic Carcinoma Cells were maintained in RPMI, 10%-FBS, 1% GPS, and 250 nM Dexamethasone and incubated in 5% CO_2 at 37°C incubator for 5 minutes, then dislodged and resuspended in the media.

Adhesion Assay

A 96-well plate was coated with Fibronectin (10μg/ml in PBS) or Collagen (10μg/ml in Acetic Acid) then allowed to incubate at 4°C for 24-48. After removal, PBS-BSA (2mg/ml) was added for 20 min. at 37°C. AT3.1 Dunning Rat Prostatic Carcinoma cells were trypsinized then incubated for 30 min. at 37°C in serum-free media with 3 μg/ml BC-ECF. The cells were aliquated (600 μl) into centrifuge tubes: 60 μl of 10x the desired concentration of spermine was added and 100 μl of the solution added to a well of a 96-well plate. The plate was incubated for 1 hour at 37°C. After incubation, the plate was inverted, twice washed with PBS, and read using an ELISA fluorescent analyzer.

Proliferation Assay

Approximately 10,000 cells were plated per well of a 6 well dish. After 25 hours, 0, 100nM, 100μM, 1mM, 5mM and 10mM Spermine was added. Cells were trypsinized after 12, 24, 48 and 60 hour incubations and counted on a Coulter Counter.

Chemotaxis Assay

Using a Boyden-Chamber apparatus, AT3.1 cells were allowed to migrate through an 8 micron pore filter towards either IGF-1 or LPA as a chemoattractant. Spermine, at concentrations of (100 μM, 1mM and 10mM was added to the cells just prior to addition to the Boyden chamber. After incubation for 4 hours at 37°C, the filter was removed, fixed, stained in Hematoxylin, and the filter with migrational cells counted under light microscopy at 100x.

Soft Agar Colony Formation

Noble Agar 0.6% in RPMI (2x normal concentration) with low (0.5%) and high (10%) serum, was plated on the bottom of 6-well dish; 0.3% agar in low and high serum was plated on the top with 3000 cells/well added with spermine at 0, 1mM, 5mM, 10mM. Plates were incubated at 37°C for 14 days. Colonies were counted under a light microscope at 400x.

In vivo Studies

Male Copenhagen Rats were shaved on the back 24 hours prior, then injected with $1x10^6$ prostate cancer cells (AT3.1 Dunning Line) subcutaneously below the scapulae on the right. Tumor size was monitored every day; tumor palpability was determined by

manual examination. Treated animals were fed a solution of 10 mM Spermine Tetrachloride (Sigma Chemical Corp.) Untreated animals were fed normal water through an autofeed system. Once tumors were palpable, tumor size was measured using calipers and tumor volume was calculated using the equation Volume = 0.52 x (Length x Width)^2.

Apoptosis

After day 10, tumor samples were resected from the treated and untreated animals, placed in 10% formalin overnight, then embedded in paraffin, sectioned and stained using the Apoptag kit (Oncor). The number of positively staining cells/high powered field was counted and averaged over 10 high powered fields per section, 2 sections/tumor.

Metastasis Study

After injection with 5 x 10^4 AT3.1 cells into the rat tail vein, tumors developed until day 21. Animals were autopsied; lungs were placed in 10% formalin then embedded in paraffin and sections were stained in himatoxylin and cosin.

Results

In vitro studies to determine the potential mechanisms involved in the inhibition of prostate cancer began with proliferation studies, which showed significant inhibition at the 10mM range. To analyze the potential effect of spermine on tumor initiation, we examined the ability of Dunning AT3.1 prostate carcinoma cells to adhere to fibronectin and to collagen. The results showed that at concentrations >100nM spermine blocks the carcinoma cells'

adhesion to both of these extracellular matrix components. Cell viability after spermine administration was confirmed using a trypan blue assay.

The ability of AT3.1 Dunning Prostate Cancer lines to form colonies in soft agar was inhibited in the presence of spermine. Addition of 1mM aminoguanidine, an inhibitor of sperminecatalysis by amine oxidase, was not able to block the inhibitory activity of spermine on cells grown in 3-dimensional cultures.

In order to determine the most effective initial oral dose, male Copenhagen rats were treated for 10 days with varying concentrations of spermine from 0.1 to 100mM. No weight change was noted; however, at the 30mM range, glucose was necessary for palatability of the solution. No other gross toxic effects were present on examination of the animals.

In the first set of oral administration *in vivo* tumor experiments, male rats were inoculated subcutaneously with $1x10^6$ prostate cancer cells (AT3.1 Dunning Line) and treated daily after the tumor was first palpable (usually 8 days post-inoculation) with doses of 1mM, 5mM, 10mM, 15mM spermine. Of these four doses, the data indicated that the 10mM concentration, dissolved in water, given continuously over 24 hours, optimized the inhibition of tumor growth (T/C = 0.46). In an attempt to compare the effect on once a day dosage, the animals were treated once daily with 10mM beginning on day 8 post-inoculation after palpable tumors arose (day 8). Again, the rate of tumor expansion was significantly retarded (T/C = 0.5) (Figure 7). When treatment with 10mM

Spermine was begun on the day of injection, subsequent tumor growth was completely prevented, whereas control rats developed palpable tumors within approximately 8 days (Figures 8A and 8B).

From the initial tumor group of treated versus control animals, resection of the primary tumor and sectioning followed by staining for apoptosis using the Apoptag method, showed that spermine treated tumors had a 4 fold higher number of apoptotic cells compared to control untreated tumors.

Autopsy studies of animals at day 21 show a difference in the weight of lungs, indicating an effect on metastatic seeding growth. Further autopsy studies of lungs stained after autopsy on day 14 show differential expression of micrometastasis on hematoylin and cosin staining. The results of one experiment are set forth in the table below.

Experimental Metastasis (Injected $5x10^4$ cells into rat tail vein, and feed 10 mM Spermine)				
		Lung weight	Colony #	Size
Non Treated				
	AVG	2.97	11.6	4.92
	STD	0.53	4.96	0.39
	Range	2.19-3.81	4.0-19.0	2.3-7.9
Treated				
2 days before injection	AVG	1.81	2.33	4.68
	STD	0.14	0.47	0.3
	Range	1.68-2	2.0-3.0	2.6-5.9
0 day of injection	AVG	1.73	1.75	4.55
	STD	0.27	1.09	0.29
	Range	1.51-2.19	0-3	2.7-6.4
Normal				
	AVG	1.64		
	STD	0.11		

Discussion

The proliferative capacity of prostatic carcinoma appears to be under the control of factors produced by the local microenvironment. In the primary site, prostatic carcinomas expand slowly, often taking years or decades to reach a size and stage compatible with metastatic spread (3). In contrast, metastatic colonies, especially those in the vertebral cavity, expand rapidly often leading to death within a few years. We previously reported that human bone marrow extracts contain agents that stimulate prostatic carcinoma cell growth (5,16). We now report that normal human prostate tissue contains an inhibitor of prostatic carcinoma

cell growth and that the inhibitory activity can be attributed to the presence of the polyamine spermine in the prostatic extracts. Spermine was shown previously to have cytostatic and cytotoxic effects on several cell types when tested in the presence of serum (9-12). Because spermine is more abundant in the prostate than in any other tissue (6), inhibitory concentrations of spermine are more likely to be found in this organ than in others. Based on these results, we speculate that the growth of early stage prostatic carcinoma cells may be retarded by the locally high concentrations of spermine.

The inhibitory action of spermine on cell growth has been attributed to two distinct mechanisms: generation of toxic byproducts by amine oxidases and feedback inhibition on polyamine synthetic enzymes. In the presence of serum aminoguanidine, an amine oxidase inhibitor, prevented growth inhibition by spermine *in vitro* at concentrations <1 mM but not at higher concentrations, demonstrating that spermine can inhibit prostatic carcinoma cell growth by both amine oxidase-dependent and independent pathways depending on the spermine concentrations employed.

Our results show that spermine, injected directly into prostatic carcinomas grown subcutaneously in syngeneic rats, significantly retarded tumor growth *in vivo*. AMG had no effect on this antitumoral activity suggesting a direct action of spermine on tumor growth *in vivo*. Based on the tumor size ($<$ 1 cm^3) at the time of initial inoculation, the level of spermine administered {100 μ1 of a 0.12 M solution (12 micromoles/tumor)/day} represents a concentration of approximately 10-12 mM, which is greater than the

concentration (1-5 mM) at which *in vitro* cell growth becomes amine oxidase-independent. This spermine concentration is also equivalent to that reported previously to inhibit cell growth directly by blocking expression of ornithine decarboxylase (ODC) and S-adenosyl-methionine decarboxylase (SAMDC), key enzymes in the polyamine synthetic pathway (13). Extensive work has shown that high intracellular levels of these polyamine synthetic enzymes are required for neoplastic cell growth (17-21).

We believe that the growth inhibition observed in our *in vivo* experiments has direct bearing on the situation in the prostate since the spermine level in prostatic fluid (~12 mM) (22) is similar to that achieved by our intratumoral injections. Hence, the slow growth of prostatic carcinoma at its site of origin may be dictated by the levels of spermine within the prostatic micro- environment. Escape from this regulation may reflect changes in the amount of spermine produced by the tumor cells themselves as well as the extent to which the surrounding secretory ducts have been compromised by tumor invasion. It is interesting to note that, in the absence of spermine, AMG alone had a stimulatory effect on tumor growth *in vivo*, but not *in vitro*. This suggests that inhibition of amine oxidases *in situ* may relieve some negative growth control possibly by preventing catabolism of endogenous spermine to cytotoxic byproducts.

Treatment of cells with high levels of exogenous polyamines often causes feedback inhibition of ODC and SAMDC with a consequent inhibition of cell proliferation (13). For this reason, polyamine analogs have been designed or selected that dramatically

suppress polyamine synthesis by reducing ODC and SAM activity and increase levels of polyamine catabolic enzymes such as spermidine/spermine N^1-acetyltransferase (SSAT). Some of these substances, such as difluoromethylornithine (DFMO) and N^1,N^{12} Bis-ethylspermine have been proposed and tested as antitumor agents on a variety of tumor types (23-26). Prostatic polyamine production is particularly sensitive to the action of these agents (6,23). Our current results suggest that blocking polyamine synthesis using polyamine analogs may not be advisable in patients with primary prostate tumors. If locally high concentrations of spermine in the prostate do act to contain prostatic carcinoma cell growth, then systemic treatments that lower polyamine levels may remove an endogenous brake on the growth of primary prostatic tumors.

The following references are cited throughout the specification. All documents mentioned herein are incorporated herein by reference.

1. Boring, C. C., Squires, T. S., Tong, T. & Montgomery, S. Cancer statistics, 1994. *Ca:Cancer J. Clin.* **44**, 7-26 (1994).
2. Scott, R. Jr., Mutchnik, D. L., Laskowski, T. Z. & Schmalhorst, W. R. Cancer of the prostate in elderly men: incidence, growth characteristics and clinical significance. *J. Urol.* **101**, 602-607 (1969).
3. Stamey, T. A. *et al.* Localized prostate cancer. Relationship of tumor volume to clinical significance for treatment of prostate cancer. *Cancer* **71**, (3 Suppl), 933-938 (1993).

4. McNeal, J. E. Prostatic microcarcinomas in relation to cancer origin and the evolution to clinical cancer. *Cancer* **71**, (3 Suppl), 984-991 (1993).

5. Rossi, M. C. & Zetter, B. R. Selective stimulation of prostatic carcinoma cell proliferation by transferrin. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6197-6201 (1992).

6. Heston, W. D. W. Prostatic polyamines and polyamine targeting as a new approach to therapy of prostatic cancer. *Cancer Surveys*, **11**, 217-238 (1991).

7. Mann, T. *Biochemistry of semen and of the male reproductive tract*. 193-200 (John Wiley, New York, 1964).

8. Heby, O. & Andersson, G. Simplified micro-method for the quantitative analysis of putrescine, spermidine and spermine in the urine. *J. Chromatogr.* **145**, 73-80 (1978).

9. Webber, M. M. & Chapronière-Rickenberg, D. Spermine oxidation products are selectively toxic to fibroblasts in cultures of normal human prostatic epithelium. *Cell Biol. Internat. Rep.* **4**, 185-193 (1980).

10. Allen, R. D. & Roberts, T. K. Role of spermine in the cytotoxic effects of seminal plasma. *Am. J. Repro. Immunol.* **13**, 4-8 (1987).

11. Coffino, P. & Poznanski A. Killer Polyamine? *J. Cell. Biochem.* ***45,*** 54-58 (1991).

12. Brunton, V. G., Grant, M. H. & Wallace H. M. Mechanisms of spermine toxicity in baby-hamster kidney (BHK) cells. The role of amine oxidases and oxidative stress. *Biochem. J.* **280**, 193-198 (1991).

13. Kameji, T. & Pegg, A. E. Inhibition of translation of mRNAs for ornithine decarboxylase and S-adenosylmethionine

decarboxylase by polyamines. *J. Biol. Chem.* **262**, 2427-2430 (1987).

14. Gahl, W. A. & Pitot, H. C. Reversal by aminoguanidine of the inhibition of proliferation of human fibroblasts by spermidine and spermine. *Chem. Biol. Interactions* **22**, 91-98 (1978).

15. Isaacs, J. T., Isaacs, W. B., Feitz, W. F. & Scheres, J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. *Prostate* **9**, 261-281 (1986).

16. Chackal-Roy, M., Niemeyer, C., Moore, M. & Zetter, B. R. Stimulation of human prostatic carcinoma cell growth by factors present in human bone marrow. *J. Clin. Invest.* **84**, 43-50 (1989).

17. Celano, P., Baylin, S. B. & Casero, R., Jr. Polyamines differentially modulate the transcription of growth-associated genes in human colon carcinoma cells. *J. Biol. Chem.* **264**, 8922-8927 (1989).

18. Pegg, A. E. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.* **48**, 759-774 (1988).

19. Hibshoosh, H., Johnson, M. & Weinstein, I. B. Effects of overexpression of ornithine decarboxylase (ODC) on growth control and oncogene-induced cell transformation. *Oncogene* **6**, 739-743 (1991).

20. Moshier, J. A., Dosescu, J., Skunca, M. & Luk, G. D. Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. *Cancer Res.* **53**, 2618-2622 (1993).

21. Auvinen, M., Paasinen, A., Andersson, L. C. & Holtta, E. Ornithine decarboxylase activity is critical for cell transformation. *Nature* **360**, 355-358 (1992).

22. Zaneveld, L. J. D. & Tauber, P. F. *The Prostatic Cell: Structure and Function* Part A 265-277 (Alan R. Liss, New York, 1981).

23. Bernacki, R. J., Bergeron, R. J. & Porter, C. W. Antitumor activity of N, N'-Bis(ethyl)spermine homologues against Human MALME-3 melanoma xenografts. *Cancer Res.* **52**, 2424-2430 (1992).

24. Porter, C. W., Bernacki, R. J., Miller, J. & Bergeron, R. J. Antitumor activity of N^1, N^{11}-bis(ethyl)norspermine against human melanoma xenografts and possible biochemical correlates of drug action. *Cancer Res.* **53**, 581-586 (1993).

25. Davidson, N. E., Mank, A. R., Prestigiacomo, L. J., Bergeron, R. J. & Casero, R. A. Growth inhibition of hormone-responsive and resistant human breast cancer cells in culture by N^1, N^{12}-Bis(ethyl)spermine. *Cancer Res.* **53**, 2071-2075 (1993).

26. Danzin, C., Jung, M. J., Grove, J. & Bey, P. Effect of alpha-difluoromethylornithine, an enzyme-activated inhibitor of ornithine decarboxylase, on polyamine levels in rat tissues. *Life Sci.* **24**, 519-524 (1979).

27. Janne, J., Raina A. & Sjimes, M. Spermidine and spermine in rat tissues at different ages. *Acta Physiol. Scand.* **62**, 352-358 (1964).

28. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. & Mitchell, J. B. Evaluation of a tetrazolium-based

semiautomated colorimetric assay: assessment of hemosensitivity testing. *Cancer Res.* **47**, 936-942 (1987).

29. Kabra, P. M., Lee, H. K., Lubich, W. P. & Morton, L. J. Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. *J. Chromatogr.* **380**, 19-32 (1986).

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. A method of treating prostate cancer in a human comprising administering to said human an effective treatment amount of spermine.

2. The method of claim 1, wherein the spermine is administered to said human after discovery or removal of a primary prostate tumor.

3. The method of claim 1, wherein said human is suffering from a metastatic disease.

4. The method of claim 1, 2 or 3 wherein the spermine is administered orally.

5. The method of claim 1, wherein the amount of spermine administered is from about 1 μg to about 10g per kilogram body weight of recipient per day

6. The method of claim 1, wherein the amount of spermine administered is from about 10 mg to about 5g per kilogram body weight of recipient per day.

7. The method of claim 1, wherein the amount of spermine administered is from about 100 to about 1000 mg per kilogram body weight of recipient per day.

8. A method for inducing cell apoptosis *in vivo* which comprises contacting a human prostate cell with an effective amount of spermine.

9. The method of claim 6, wherein the cell is a human prostate cancer cell.

10. A method of treating benign prostate hyperplasia in a human comprising administering to said human an effective treatment amount of spermine.

11. A pharmaceutical composition comprising an effective amount of spermine and a pharmaceutically acceptable carrier.

12. The pharmaceutical composition of claim 11, wherein said composition is adapted for oral administration.

13. The pharmaceutical composition of claim 12, wherein the amount of spermine is from 50 mg to 1000 mg.

14. A method of prophylaxis in a member of a population at risk for prostate cancer, comprising administering an effective amount of spermine to said member.

15. The method of claim 14, wherein the member is selected from the group consisting of one having a family history of prostate cancer, one having a high prostate-specific antigen (PSA) level, or one who has had an increase in PSA levels.

16. Use of a pharmaceutical composition containing spermine in the manufacture of a medicament for treating prostate cancer.

17. A method for determining whether administration of an agent that blocks or inhibits polyamine synthesis is an appropriate treatment in a subject, comprising screening said subject for the presence of a primary prostate tumor prior to the administration of the agent that blocks or inhibits polyamine synthesis, wherein the presence of primary prostate tumor indicates that the prolonged administration of the agent is contraindicated.

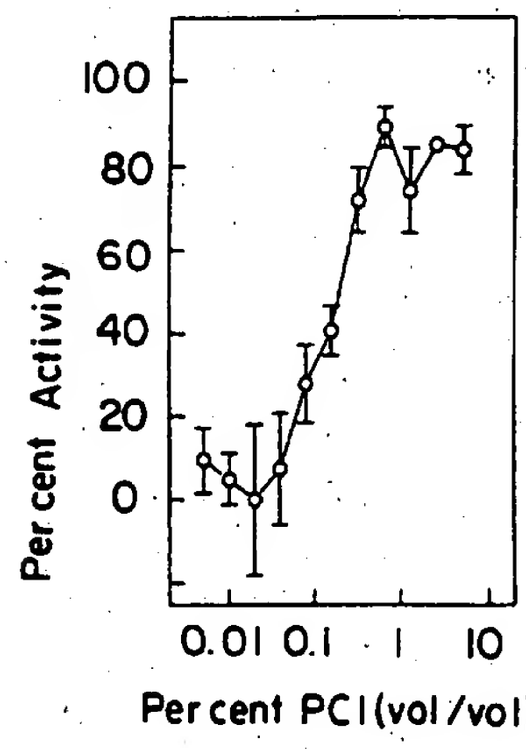

FIG. 1A

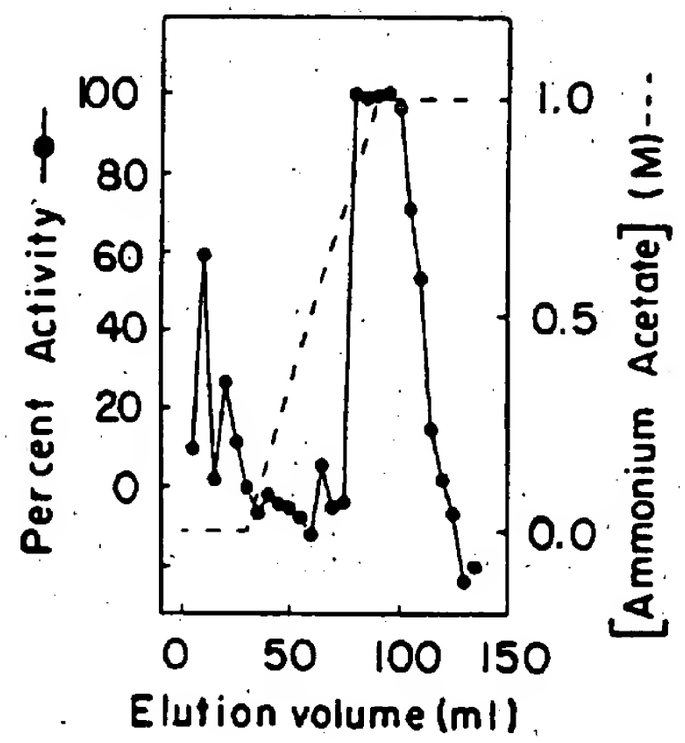

FIG. 1B

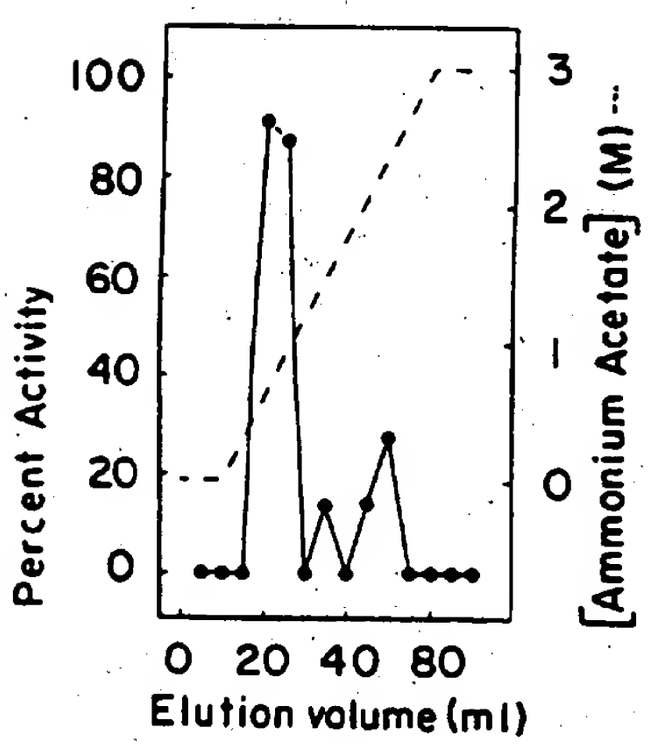

FIG. 1C

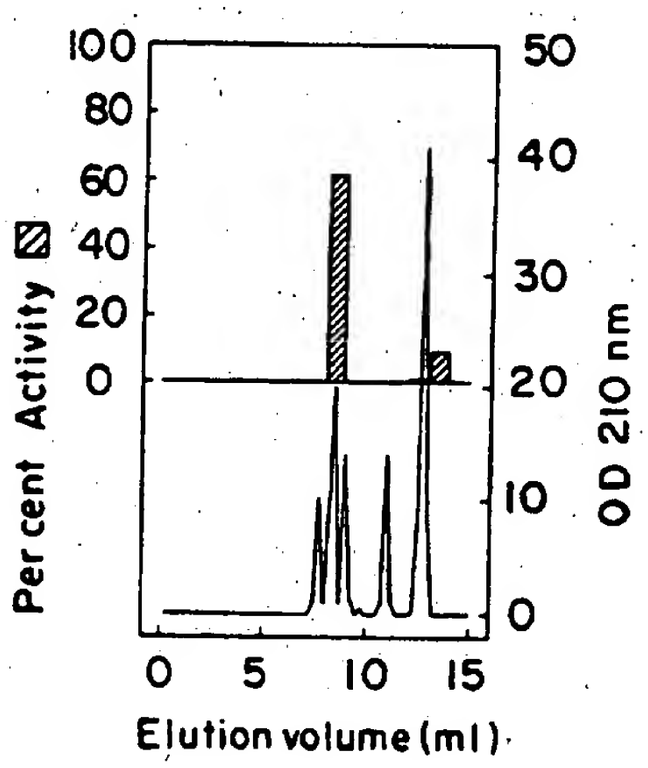

FIG. 1D

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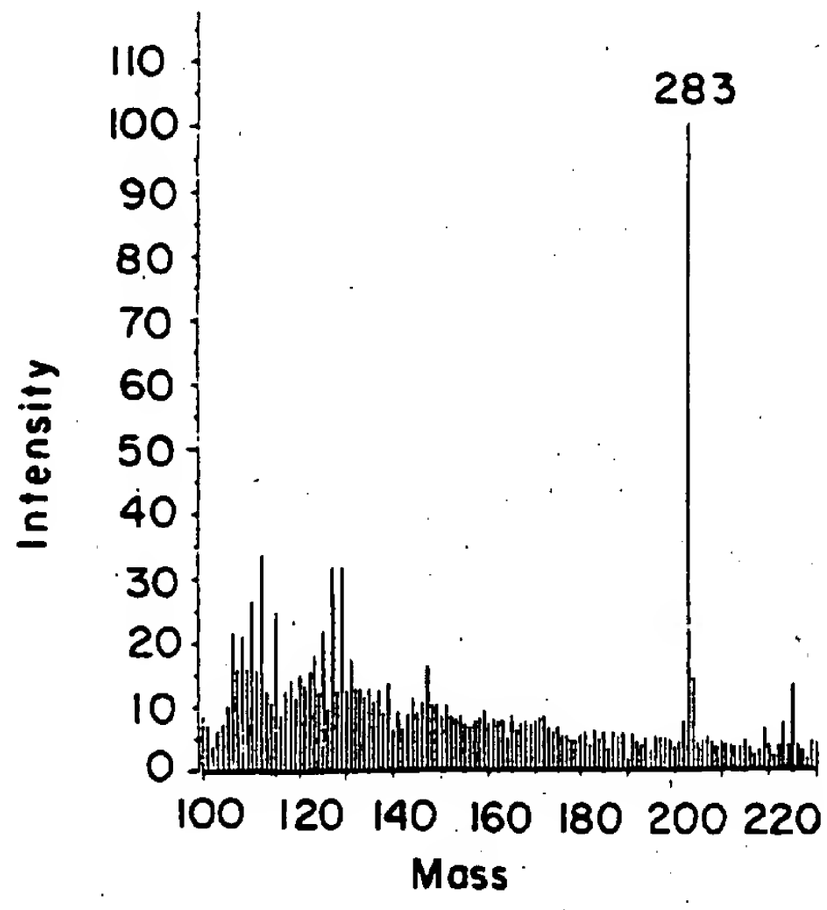

FIG. 2A

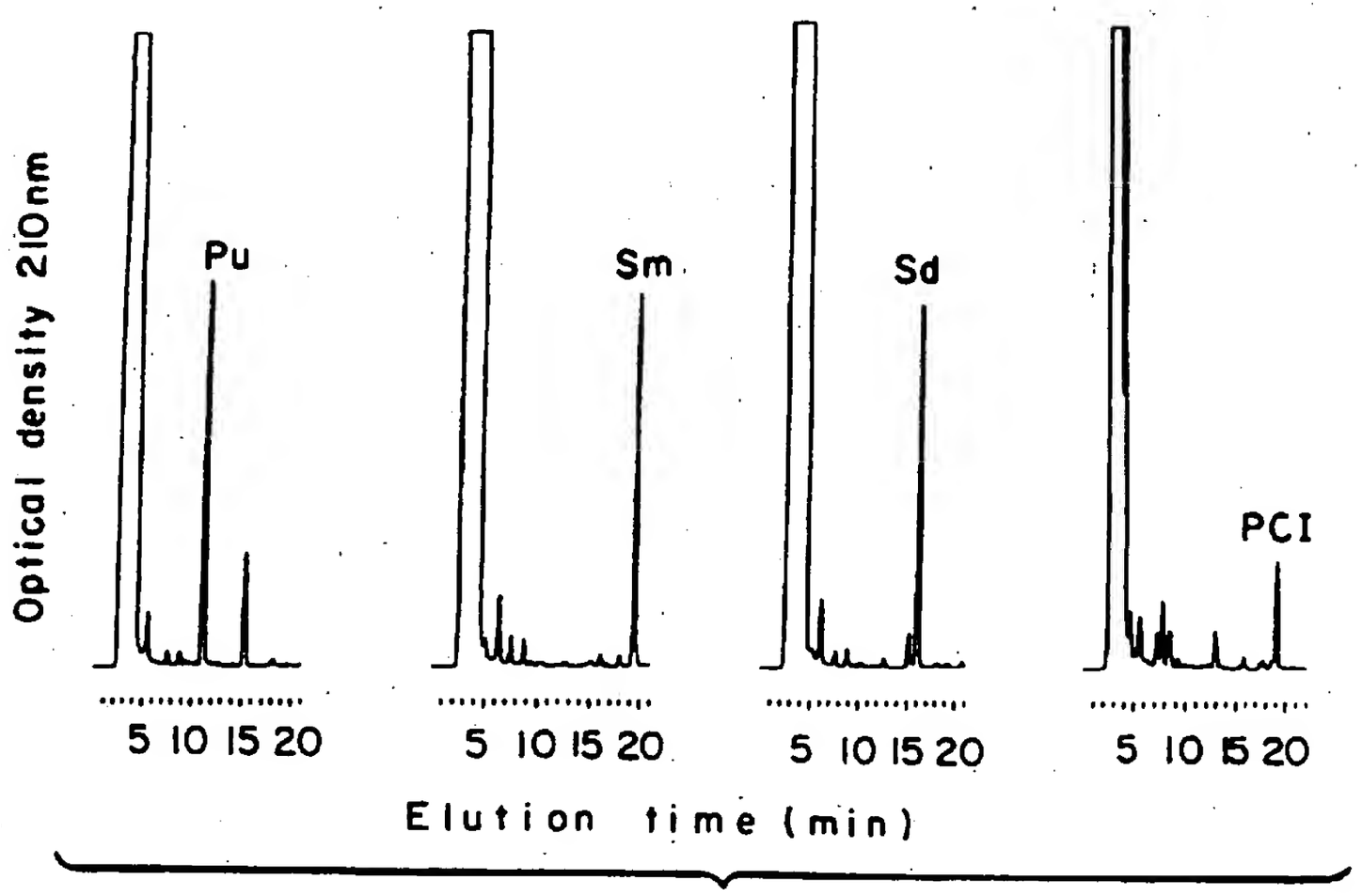

FIG. 2B

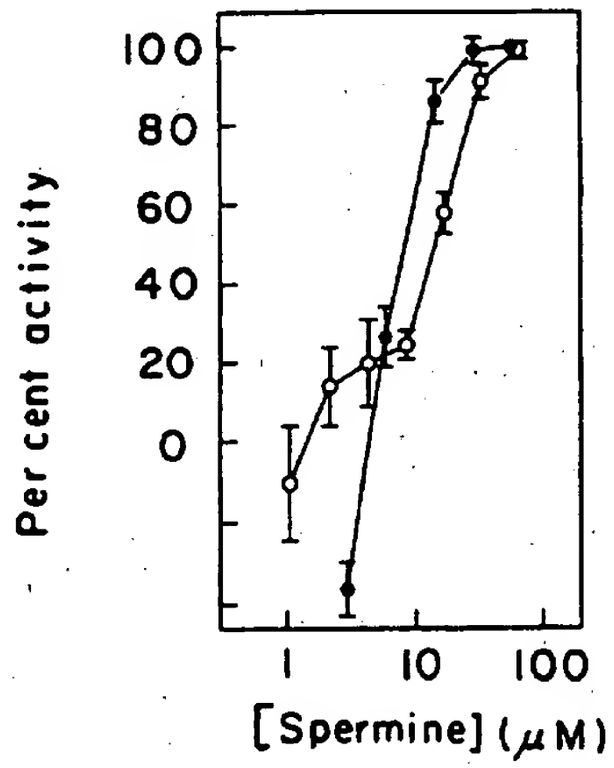

FIG. 3A

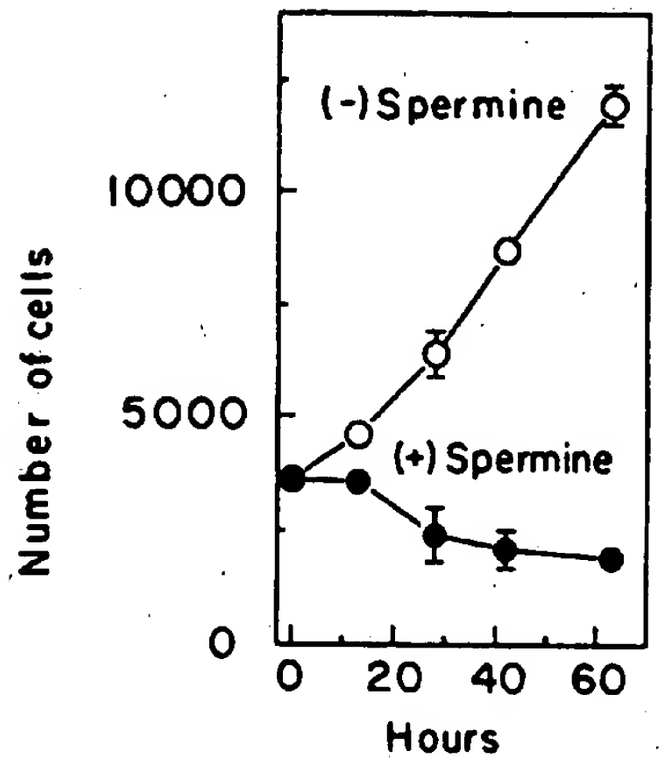

FIG. 3B

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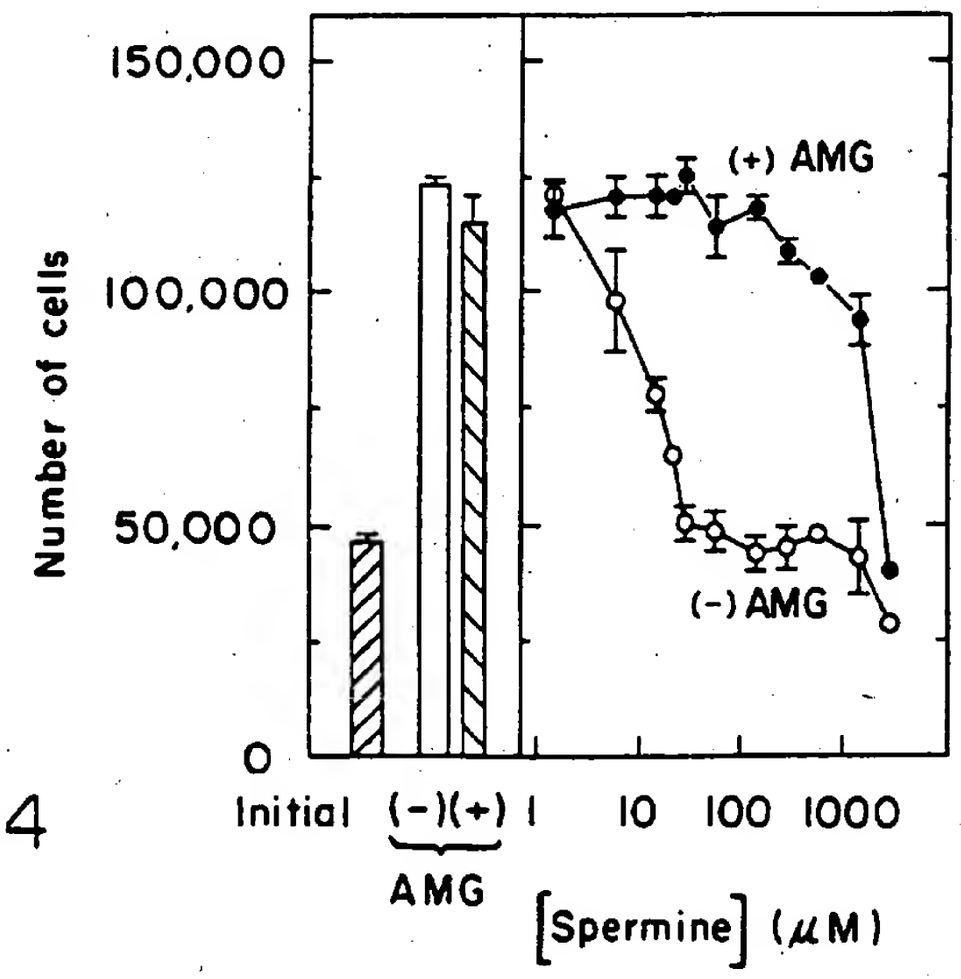


FIG. 4

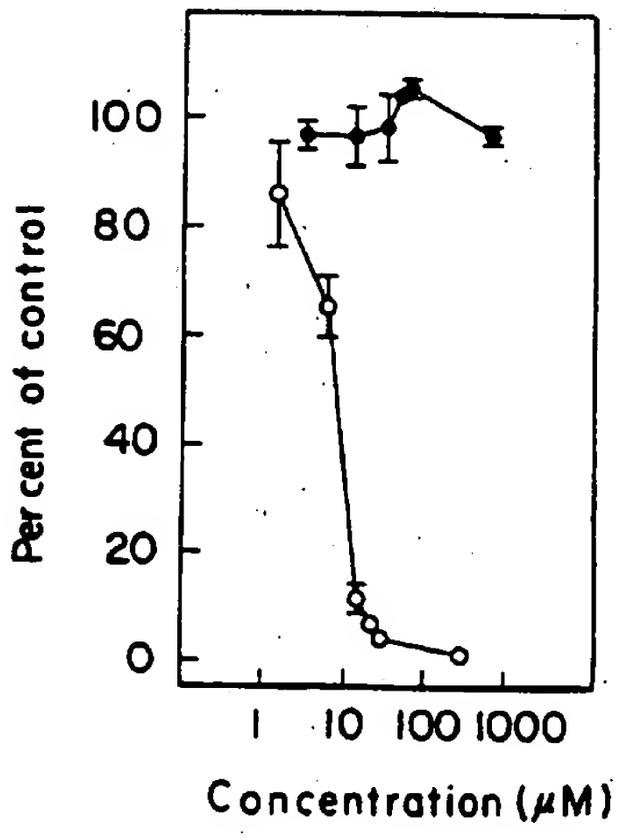


FIG. 5

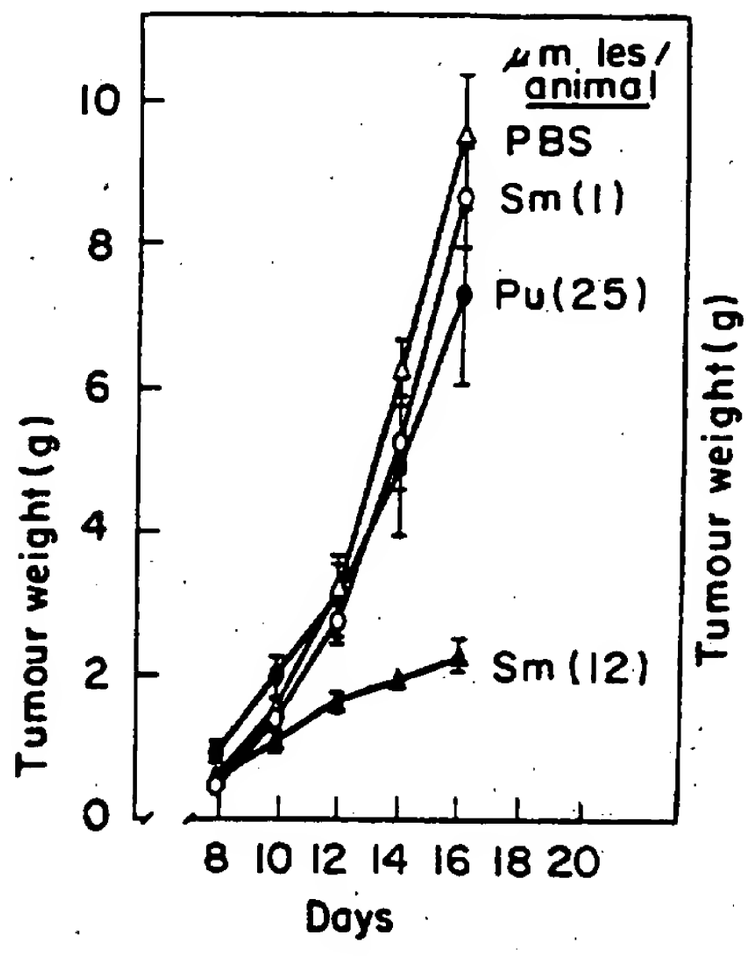


FIG. 6A

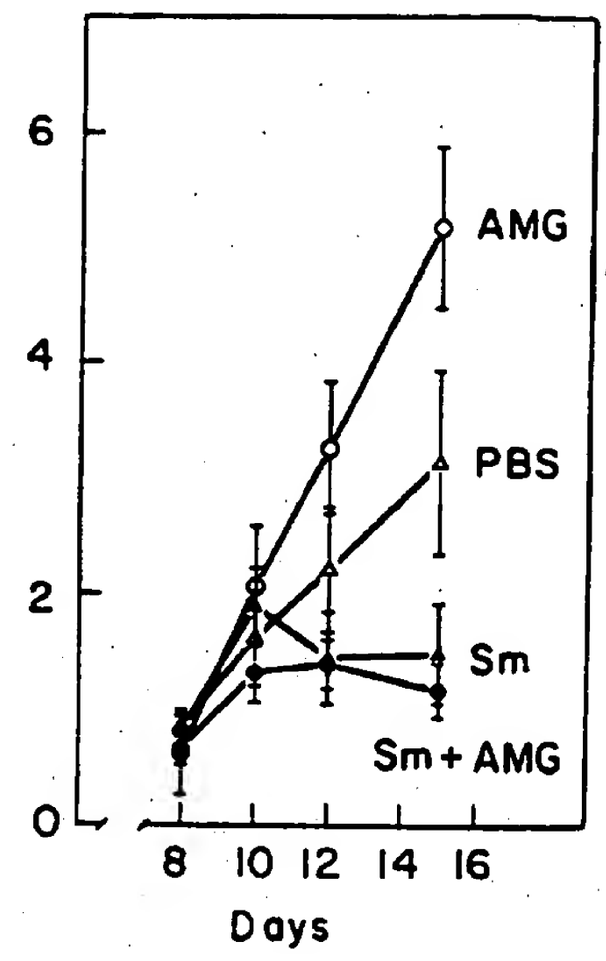


FIG. 6B

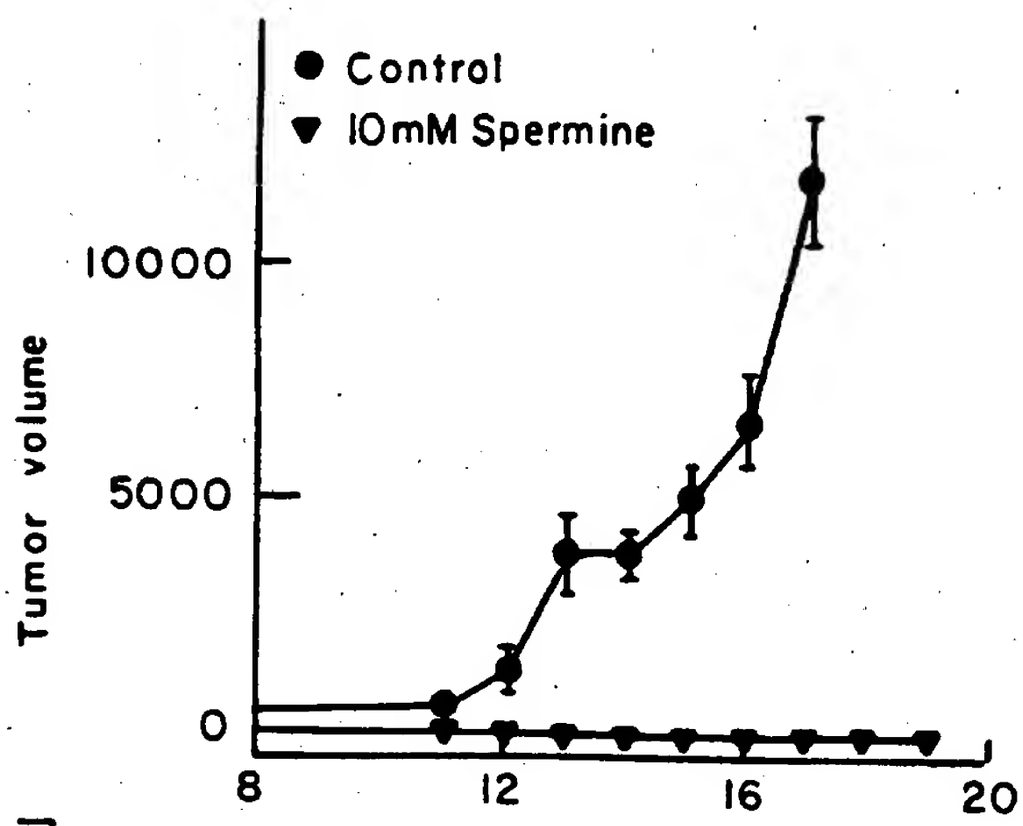


FIG. 7

FIG.8A

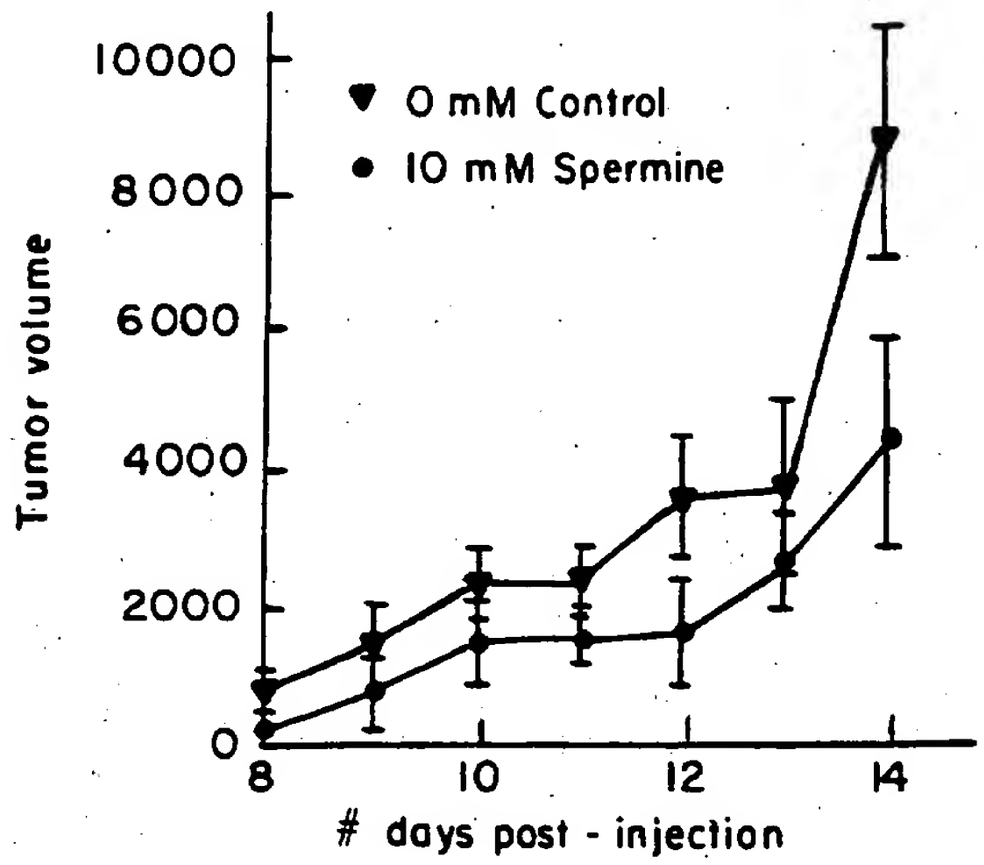
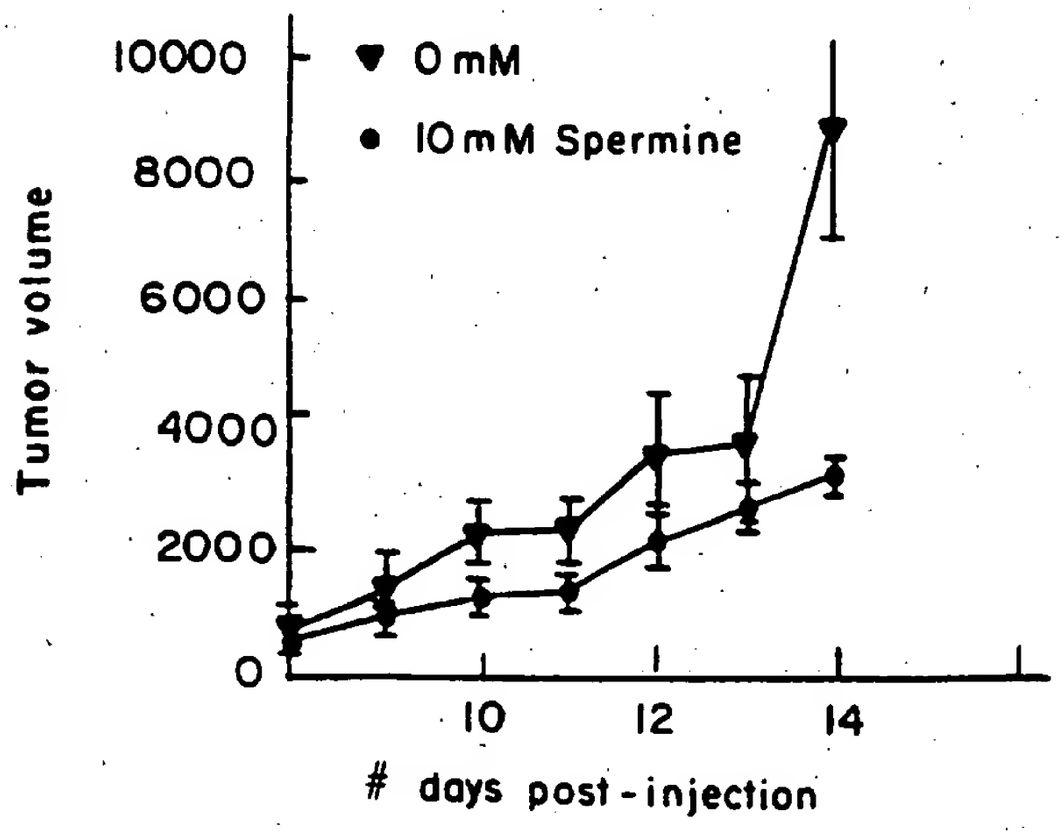


FIG.8B



INTERNATIONAL SEARCH REPORT

Inv ional Application No
PCT/US 96/15626

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. ANNU. MEET. AM. ASSOC. CANCER RES., vol. 35, 1994, page 284 XP000616210 R.C. SMITH ET AL.: "Isolation and characterization of a low molecular weight inhibitor of prostatic adenocarcinoma from human prostate: spermine as an in situ growth modulator of the primary tumor." cited in the application abstract # 1694 -----	

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
21 January 1997	31.01.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Klaver, T

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